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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		TSRI 578.2
		U.S. APPLICATION NO. (If known, see 37 CFR 1 5) 09/341550
INTERNATIONAL APPLICATION NO. PCT/US98/00840	INTERNATIONAL FILING DATE 16 January 1998	PRIORITY DATE CLAIMED 17 January 1997

TITLE OF INVENTION
RNA BINDING PROTEIN AND BINDING SITE USEFUL FOR EXPRESSION OF RECOMBINANT MOLECULES

APPLICANT(S) FOR DO/EO/US
Stephen Mayfield

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [XX] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [XX] Other items or information:
    - Return Receipt Postcard
    - Certificate of Express Mailing

80 Rec'd PCT/PTO 13 JUL 1999

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 09/341550	INTERNATIONAL APPLICATION NO PCT/US98/00840	ATTORNEY'S DOCKET NUMBER TSRI 578.2

	CALCULATIONS	PTO USE ONLY
17. [X] The following fees are submitted: **BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):** Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO .... **$970.00** International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO .... **$840.00** International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO .... **$760.00** International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) .... **$670.00** International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 96.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	65 - 20 =	45	X $18.00	$ 810.00	
Independent claims	31 - 3 =	28	X $78.00	$ 2184.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $260.00	$	

TOTAL OF ABOVE CALCULATIONS =	$ 2994.00	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).	$	
SUBTOTAL =	$ 3090.00	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$	
TOTAL NATIONAL FEE =	$ 3090.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$ 40.00	
TOTAL FEES ENCLOSED =	$ 3130.00	
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	charged	$

a. [XX] A check in the amount of $3090 & 40.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. ____________ in the amount of $____________ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [XX] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0962. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

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40,652
REGISTRATION NUMBER

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09/341550

RNA BINDING PROTEIN AND BINDING SITE USEFUL FOR EXPRESSION OF RECOMBINANT MOLECULES

Technical Field

The invention relates to expression systems and methods for expression of desired genes and gene products in cells. Particularly, the invention relates to a gene encoding a RNA binding protein useful for regulating gene expression in cells, the protein binding site, a gene encoding a regulating protein disulfide isomerase and methods and systems for gene expression of recombinant molecules.

Background

Expression systems for expression of exogenous foreign genes in eukaryotic and prokaryotic cells are basic components of recombinant DNA technology. Despite the abundance of expression systems and their wide-spread use, they all have characteristic disadvantages. For example, while expression in *E. coli* is probably the most popular as it is easy to grow and is well understood, eukaryotic proteins expressed therein are not properly modified. Moreover, those proteins tend to precipitate into insoluble aggregates and are difficult to obtain in large amounts. Mammalian expression systems, while practical on small-scale protein production, are more difficult, time-consuming and expensive than in *E. coli*.

A number of plant expression systems exist as well as summarized in US Patent No. 5,234,834, the disclosures of which are hereby incorporated by reference. One advantage of plants or algae in an expression system is that they can be used to produce pharmacologically important proteins and enzymes on a large scale and in relatively pure form. In addition, micro-algae have several unique characteristics that make them ideal organisms for the production of proteins on a large scale.

First, unlike most systems presently used to produce transgenic proteins, algae can be grown in minimal media (inorganic salts) using sunlight as the energy source. These algae can be grown in contained fermentation vessels or on large scale in monitored ponds. Ponds of up to several acres are routinely used for the production of micro-algae. Second, plants and algae have two distinct compartments, the cytoplasm and the chloroplast, in which proteins can be expressed. The cytoplasm of algae is similar to that of other eukaryotic organisms used for protein expression, like yeast and insect cell cultures. The chloroplast is unique to plants and algae and proteins expressed in this environment are likely to have properties different from those of cytoplasmically expressed proteins.

The present invention describes an expression system in which exogenous molecules are readily expressed in either prokaryotic or eukaryotic hosts and in either the cytoplasm or chloroplast. These beneficial attributes are based on the discovery and cloning of components of translation regulation in plants as described in the present invention.

Protein translation plays a key role in the regulation of gene expression across the spectrum of organisms (Kozak, Ann. Rev. Cell Biol., 8:197-225 (1992) and de Smit and Van Duin, Prog. Nucleic Acid Res. Mol. Biol., 38:1-35 (1990)). The majority of regulatory schemes characterized to date involve translational repression often involving proteins binding to mRNA to limit ribosome association (Winter et al., Proc. Natl. Acad. Sci., USA, 84:7822-7826 (1987) and Tang and Draper, Biochem., 29:4434-4439 (1990)). Translational activation has also been observed (Wulczyn and Kahmann, Cell, 65:259-269 (1991)), but few of the underlying molecular mechanisms for this type of regulation have been identified. In plants, light

activates the expression of many genes. Light has been shown to activate expression of specific chloroplast encoded mRNAs by increasing translation initiation (Mayfield et al., Ann. Rev. Plant Physiol. Plant Mol. Biol., 46:147-166 (1995) and Yohn et al., Mol. Cell Biol., 16:3560-3566 (1996)). Genetic evidence in higher plants and algae has shown that nuclear encoded factors are required for translational activation of specific chloroplast encoded mRNAs (Rochaix et al., Embo J., 8:1013-1021 (1989), Kuchka et al., Cell, 58:869-876 (1989), Girard-Bascou et al., Embo J., 13:3170-3181 (1994), Kim et al., Plant Mol. Biol., 127:1537-1545 (1994).

In the green algae *Chlamydomonas reinhardtii*, a number of nuclear mutants have been identified that affect translation of single specific mRNAs in the chloroplast, often acting at translation initiation (Yohn et al., supra, (1996)). Mutational analysis of chloroplast mRNAs has identified sequence elements within the 5' untranslated region (UTR) of mRNAs that are required for translational activation (Mayfield et al., supra, (1995), Mayfield et al., J. Cell Biol., 127:1537-1545 (1994) and Rochaix, Ann. Rev. Cell Biol., 8:1-28 (1992)), and the 5' UTR of a chloroplast mRNA can confer a specific translation phenotype on a reporter gene *in vivo* (Zerges and Rochaix, Mol. Cell Biol., 14:5268-5277 (1994) and Staub and Maliga, Embo J., 12:601-606 (1993).

Putative translational activator proteins were identified by purifying a complex of four proteins that binds with high affinity and specificity to the 5' UTR of the chloroplast encoded *psb*A mRNA [encoding the D1 protein, a major component of Photosystem II (PS II)] (Danon and Mayfield, Embo J., 10:3993-4001 (1991)). Binding of these proteins to the 5' UTR of *psb*A mRNA correlates with translation of this mRNA under a variety of

physiological (Danon and Mayfield, id., (1991)) and biochemical conditions (Danon and Mayfield, Science, 266:1717-1719 (1994) and Danon and Mayfield, Embo J., 13:2227-2235 (1994)), and in different genetic backgrounds (Yohn et al., supra, (1996)). The binding of this complex to the *psb*A mRNA can be regulated *in vitro* in response to both redox potential (Danon and Mayfield, Science, 266:1717-1719 (1994)) and phosphorylation (Danon and Mayfield, Embo J., 13:2227-2235 (1994)), both of which are thought to transduce the light signal to activate translation of *psb*A mRNA. The 47 kDa member of the *psb*A RNA binding complex (RB47) is in close contact with the RNA, and antisera specific to this protein inhibits binding to the *psb*A mRNA *in vitro* (Danon and Mayfield, supra, (1991)).

Although the translational control of psbA mRNA by RB47 has been reported, the protein has not been extensively characterized and the gene encoding RB47 has not been identified, cloned and sequenced. In addition, the regulatory control of the activation of RNA binding activity to the binding site by nuclear-encoded *trans*-acting factors, such as RB60, have not been fully understood. The present invention now describes the cloning and sequencing of both RB47 and RB60. Based on the translation regulation mechanisms of RB47 and RB60 with the RB47 binding site, the present invention also describes a translation regulated expression system for use in both prokaryotes and eukaryotes.

Brief Description of the Invention

The RB47 gene encoding the RB47 activator protein has now been cloned and sequenced, and the target binding site for RB47 on messenger RNA (mRNA) has now been identified. In addition, a regulatory protein disulfide isomerase, a 60 kilodalton protein

referred to as RB60, has also been cloned, sequenced and characterized. Thus, the present invention is directed to gene expression systems in eukaryotic and prokaryotic cells based on translational regulation by RB47 protein, its binding site and the RB60 regulation of RB47 binding site activation.

More particularly, the present invention describes the use of the RB47 binding site, i.e., a 5' untranslated region (UTR) of the chloroplast *psb*A gene, in the context of an expression system for regulating the expression of genes encoding a desired recombinant molecule. Protein translation is effected by the combination of the RB47 binding site and the RB47 binding protein in the presence of protein translation components. Regulation can be further imposed with the use of the RB60 regulatory protein disulfide isomerase. Therefore, the present invention describes reagents and expression cassettes for controlling gene expression by affecting translation of a coding nucleic acid sequence in a cell expression system.

Thus, in one embodiment, the invention contemplates a RB47 binding site sequence, i.e., a mRNA sequence, typically a mRNA leader sequence, which contains the RB47 binding site. A preferred RB47 binding site is *psb*A mRNA. For use in expressing recombinant molecules, the RB47 binding site is typically inserted 5' to the coding region of the preselected molecule to be expressed. In a preferred embodiment, the RB47 binding site is inserted into the 5' untranslated region along with an upstream *psb*A promoter to drive the expression of a preselected nucleic acid encoding a desired molecule. In alternative embodiments, the RB47 binding site is inserted into the regulatory region downstream of any suitable promoter present in a eukaryotic or prokaryotic expression vector. Preferably, the RB47 binding site is positioned within 100 nucleotides of the

translation initiation site. In a further aspect, 3' to the coding region is a 3' untranslated region (3' UTR) necessary for transcription termination and RNA processing.

Thus, in a preferred embodiment, the invention contemplates an expression cassette or vector that contains a transcription unit constructed for expression of a preselected nucleic acid or gene such that upon transcription, the resulting mRNA contains the RB47 binding site for regulation of the translation of the preselected gene transcript through the binding of the activating RB47 protein. The RB47 protein is provided endogenously in a recipient cell and/or is a recombinant protein expressed in that cell.

Thus, the invention also contemplates a nucleic acid molecule containing the sequence of the RB47 gene. The nucleic acid molecule is preferably in an expression vector capable of expressing the gene in a cell for use in interacting with a RB47 binding site. The invention therefore contemplates an expressed recombinant RB47 protein. In one embodiment, the RB47 binding site and RB47 encoding nucleotide sequences are provided on the same genetic element. In alternative embodiments, the RB47 binding site and RB47 encoding nucleotide sequences are provided separately.

The invention further contemplates a nucleic acid molecule containing the sequence encoding the 69 kilodalton precursor to RB47. In alternative embodiments, the RB47 nucleic acid sequence contains a sequence of nucleotides to encode a histidine tag. Thus, the invention relates to the use of recombinant RB47, precursor RB47, and histidine-modified RB47 for use in enhancing translation of a desired nucleic acid.

The invention further contemplates a nucleic acid molecule containing a nucleotide sequence of a polypeptide which

regulates the binding of RB47 to RB47 binding site. A preferred regulatory molecule is the protein disulfide isomerase RB60. The RB60-encoding nucleic acid molecule is preferably in an expression vector capable of expressing the gene in a cell for use in regulating the interaction of RB47 with a RB47 binding site. Thus, the invention also contemplates an expressed recombinant RB60 protein. In one embodiment, the RB47 binding site, RB47 encoding and RB60 encoding nucleotide sequences are provided on the same genetic element. In alternative embodiments, the expression control nucleotide sequences are provided separately. In a further aspect, the RB60 gene and RB47 binding site sequence are provided on the same construct.

The invention can therefore be a cell culture system, an *in vitro* expression system or a whole tissue, preferably a plant, in which the transcription unit is present that contains the RB47 binding site and further includes a (1) transcription unit capable of expressing RB47 protein or (2) the endogenous RB47 protein itself for the purpose of enhancing translation of the preselected gene having an RB47 binding site in the mRNA. Preferred cell culture systems are eukaryotic and prokaryotic cells. Particularly preferred cell culture systems include plants and more preferably algae.

A further preferred embodiment includes (1) a separate transcription unit capable of expressing a regulatory molecule, preferably RB60 protein, or (2) the endogenous RB60 protein itself for the purpose of regulating translation of the preselected gene having an RB47 binding site in the mRNA. In an alternative preferred embodiment, one transcription unit is capable of expressing both the RB47 and RB60 proteins. In a further aspect, the RB47 binding site sequence and RB60 sequence are provided on the same construct.

In one aspect of the present invention, plant cells endogenously containing RB47 and RB60 proteins are used for the expression of recombinant molecules, such as proteins or polypeptides, through activation of the RB47 binding in an exogenously supplied expression cassette. Alternatively, stable plant cell lines containing endogenous RB47 and RB60 are first generated in which RB47 and/or RB60 proteins are overexpressed. Overexpression is obtained preferably through the stable transformation of the plant cell with one or more expression cassettes for encoding recombinant RB47 and RB60. In a further embodiment, stable cell lines, such as mammalian or bacterial cell lines, lacking endogenous RB47 and/or RB60 proteins are created that express exogenous RB47 and/or RB60.

Plants for use with the present invention can be a transgenic plant, or a plant in which the genetic elements of the invention have been introduced. Based on the property of controlled translation provided by the combined use of the RB47 protein and the RB47 binding site, translation can be regulated for any gene product, and the system can be introduced into any plant species. Similarly, the invention is useful for any prokaryotic or eukaryotic cell system.

Methods for the preparation of expression vectors is well known in the recombinant DNA arts, and for expression in plants is well known in the transgenic plant arts. These particulars are not essential to the practice of the invention, and therefore will not be considered as limiting.

The invention allows for high level of protein synthesis in plant chloroplasts and in the cytoplasm of both prokaryotic and eukaryotic cells. Because the chloroplast is such a productive plant organ, synthesis in chloroplasts is a preferred site of translation by virtue of the large amounts of protein that can

be produced. This aspect provides for great advantages in agricultural production of mass quantities of a preselected protein product.

The invention further provides for the ability to screen for agonists or antagonists of the binding of RB47 to the RB47 binding site using the expression systems as described herein. Antagonists of the binding are useful in the prevention of plant propagation.

Also contemplated by the present invention is a screening assay for agonists or antagonists of RB60 in a manner analogous to that described above for RB47. Such agonists or antagonists would be useful in general to modify expression of RB60 as a way to regulate cellular processes in a redox manner.

Kits containing expression cassettes and expression systems, along with packaging materials comprising a label with instructions for use, as described in the claimed embodiments are also contemplated for use in practicing the methods of this invention.

Other uses will be apparent to one skilled in the art in light of the present disclosures.

Brief Description of Drawings

In the figures forming a portion of this disclosure:

Figures 1A-1D show the complete protein amino acid residue sequence of RB47 is shown from residues 1-623, together with the corresponding nucleic acid sequence encoding the RB47 sequence, from base 1 to base 2732. The nucleotide coding region is shown from base 197-2065, the precursor form. The mature form is from nucleotide position 197-1402. Also shown is the mRNA leader, bases 1-196, and poly A tail of the mRNA, bases 2066-2732. Both the nucleotide and amino acid sequence are listed in SEQ ID NO

PCT/US 98/00840
IPEA/US 17 AUG 1998

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AMENDED SHEET

PCT/US 98/00840

IPEA/US 17 AUG 1998

5.

Figures 2A-2B show the complete protein amino acid residue sequence of RB60 is shown from residues 1-488, together with the corresponding nucleic acid sequence from base 1 to base 2413, of which bases 16-1614 encode the RB60 sequence. Both the nucleotide and amino acid sequence are listed in SEQ ID NO 10.

Figures 3A-3C show the complete sequence of the psbA mRNA, showing both encoded psbA protein amino acid residue sequence (residues 1-352) and the nucleic acid sequence as further described in Example 3 is illustrated. Both the nucleotide and amino acid sequence are listed in SEQ ID NO 13.

Figure 4 is a schematic diagram of an expression cassette containing on one transcription unit from 5' to 3', a promoter region derived from the *psb*A gene for encoding the D1 protein from *C. reinhardtii* further containing a transcription initiation site (TS), the RB47 binding site, a region for insertion of a foreign or heterologous coding region, a RB47 coding region, a RB60 coding region, and the 3' flanking region containing transcription termination site (TS), flanked by an origin of replication and selection marker. Restriction endonuclease sites for facilitating insertion of the independent genetic elements are indicated and further described in Example 4A.

Figures 5A-5B show the nucleotide and amino acid sequence of the RB47 molecule containing a histidine tag, the sequences of which are also listed in SEQ ID NO 14.

Figure 6 is a schematic diagram of an expression cassette containing on one transcription unit from 5' to 3', a promoter region derived from the *psb*A gene for encoding the D1 protein from *C. reinhardtii* further containing a transcription initiation site (TS), the RB47 binding site, a region for

PCT/US 98/0084

IPEA/US 17 AUG 1998

RB47 is also shown in Figures 1A-1D (SEQ ID NO 5). As described in Section 2 above, the predicted protein sequence from the cloned cDNA contained both the derived peptide sequences of RB47 and is highly homologous to poly(A) binding proteins (PABP) from a variety of eukaryotic organisms.

2. Cloning of RB60

To clone the cDNA encoding the 60 kDa *psb*A mRNA binding protein (RB60), the *psb*A-specific RNA binding proteins were purified from light-grown *C. reinhardtii* cells using heparin-agarose chromatography followed by *psbA* RNA affinity chromatography (RAC). RAC-purified proteins were separated by two-dimensional polyacrylamide gel electrophoresis. The region corresponding to RB60 was isolated from the PVDF membrane. RB60 protein was then digested with trypsin. Unambiguous amino acid sequences were obtained from two peptide tryptic fragments (WFVDGELASDYNGPR (SEQ ID NO 6) and (QLILWTTADDLKADAEIMTVFR (SEQ ID NO 7)) as described above for RB47. The calculated molecular weights of the two tryptic peptides used for further analysis precisely matched with the molecular weights determine by mass spectrometry. The DNA sequence corresponding to one peptide of 22 amino acid residues was amplified by PCR using degenerate oligonucleotides, the forward primer 5'CGCGGATCCGAYGCBGAGATYATGAC3' (SEQ ID NO 8) and the reverse primer 5'CGCGAATTCGTCATRATCTCVGCRTC3' (SEQ ID NO 9), where R can be A or G (the other IUPAC nucleotides have been previously defined above). The amplified sequence was then used to screen a λ-gt10 cDNA library

AMENDED SHEET

PCT/US 98/00840
IPEA/US 17 AUG 1998

from *C. reinhardtii*. Three clones were identified with the largest being 2.2 kb. Selection and sequencing was performed as described for RB47 cDNA.

The resulting RB60 cDNA sequence is available via GenBank (Accession Number AF027727). The nucleotide and encoded amino acid sequence of RB60 is also shown in Figures 2A-2B (SEQ ID NO 10). The protein coding sequence of 488 amino acid residues corresponds to nucleotide positions 16-1614 of the 2413 base pair sequence. The predicted amino acid sequence of the cloned cDNA contained the complete amino acid sequences of the two tryptic peptides. The amino acid sequence of the encoded protein revealed that it has high sequence homology to both plant and mammalian protein disulfide isomerase (PDI), and contains the highly conserved thioredoxin-like domains with -CysGlyHisCys- (-CGHC-) (SEQ ID NO 11) catalytic sites in both the N-terminal and C-terminal regions and the -LysAspGluLeu- (-KDEL-) (SEQ ID NO 12) endoplasmic reticulum (ER) retention signal at the C-terminus found in all PDIs. PDI is a mutifunctional protein possessing enzymatic activities for the formation, reduction, and isomerization of disulfide bonds during protein folding, and is typically found in the ER. The first 30 amino acid residues of RB60 were found to lack sequence homology with the N-terminal signal sequence of PDI from plants or mammalian cells. However, this region has characteristics of chloroplast transit peptides of *C. reinhardtii*, which have similarities with both mitochondrial and higher plant chloroplast presequences. A transit peptide sequence should override the function

AMENDED SHEET

of the -KDEL- ER retention signal and target the protein to the chloroplast since the -KDEL- signal acts only to retain the transported protein in the ER.

3. Preparation of *psb*A Promoter Sequence and RB47 Binding Site Nucleotide Sequence

The chloroplast *psb*A gene from the green unicellular alga *C. reinhardii* was cloned and sequenced as described by Erickson et al., Embo J., 3:2753-2762 (1984), the disclosure of which is hereby incorporated by reference. The DNA sequence of the coding regions and the 5' and 3' untranslated (UTR) flanking sequences of the *C. reinhardii psb*A gene is shown in Figures 3A-3C. The *psb*A gene sequence is also available through GenBank as further discussed in Example 4. The nucleotide sequence is also listed as SEQ ID NO 13. The deduced amino acid sequence (also listed in SEQ ID NO 13) of the coding region is shown below each codon beginning with the first methionine in the open reading frame. Indicated in the 5' non-coding sequence are a putative Shine-Dalgarno sequence in the dotted box, two putative transcription initiation sites determined by S1 mapping (S1) and the Pribnow-10 sequence in the closed box. Inverted repeats of eight or more base pairs are marked with arrows and labeled A-D. A direct repeat of 31 base pairs with only two mismatches is marked with arrows labeled 31. Indicated in the 3' non-coding sequence is a large inverted repeat marked by a forward arrow and the SI cleavage site marking the 3' end of the mRNA. Both the 5' and 3' untranslated regions are used in preparing one of the expression cassettes of this

PCT/US 98/00840
IPEA/US 17 AUG 1998

invention as further described below.

The 5' UTR as previously discussed contains both the *psb*A promoter and the RB47 binding site. The nucleotide sequence defining the *psb*A promoter contains the region of the *psb*A DNA involved in binding of RNA polymerase to initiate transcription. The -10 sequence component of the *psb*A promoter is indicated by the boxed nucleotide sequence upstream of the first S1 while the -35 sequence is located approximately 35 bases before the putative initiation site. As shown in Figures 3A-3C, the -10 sequence is boxed, above which is the nucleotide position (-100) from the first translated codon. The -35 sequence is determined accordingly. A *psb*A promoter for use in an expression cassette of this invention ends at the first indicated S1 site (nucleotide position -92 as counting from the first ATG) in Figures 3A-3C and extends to the 5' end (nucleotide position -251 as shown in Figures 3A-3C). Thus, the promoter region is 160 bases in length. A more preferred promoter region extends at least 100 nucleotides to the 5' end from the S1 site. A most preferred region contains nucleotide sequence ending at the s1 site and extending 5' to include the -35 sequence, i.e., from -92 to -130 as counted from the first encoded amino acid residue (39 bases).

The *psb*A RB47 binding site region begins at the first S1 site as shown in Figures 3A-3C and extends to the first adenine base of the first encoded methionine residue. Thus, a *psb*A RB47 binding site in the *psb*A gene corresponds to the nucleotide positions from -91 to -1 as shown in Figure 3A-3C.

AMENDED SHEET

The above-identified regions are used to prepare expression constructs as described below. The promoter and RB47 binding site regions can be used separately; for example, the RB47 binding site sequence can be isolated and used in a eukaryotic or prokaryotic plasmid with a non-*psb*A promoter. Alternatively, the entire *psb*A 5' UTR having 251 nucleotides as shown in Figures 3A-3C is used for the regulatory region in an expression cassette containing both the *psb*A promoter and RB47 binding site sequence as described below.

4. <u>Preparation of Expression Vectors and Expression of Coding Sequences</u>

A. <u>Constructs Containing an *psb*A Promoter, an RB47 Binding Site Nucleotide Sequence, a Desired Heterologous Coding Sequence, an RB47-Encoding Sequence and an RB60-Encoding Sequence</u>

Plasmid expression vector constructs, alternatively called plasmids, vectors, constructs and the like, are constructed containing various combinations of elements of the present invention as described in the following examples. Variations of the positioning and operably linking of the genetic elements described in the present invention and in the examples below are contemplated for use in practicing the methods of this invention. Methods for manipulating DNA elements into operable expression cassettes are well known in the art of molecular biology. Accordingly, variations of control elements, such as constitutive or inducible promoters, with respect to prokaryotic or

PCT/US 98/00840
IPEA/US 17 AUG 1998

eukaryotic expression systems as described in Section C. are contemplated herein although not enumerated. Moreover, the expression the various elements is not limited to one transcript producing one mRNA; the invention contemplates protein expression from more than one transcript if desired.

As such, while the examples below recite one or two types of expression cassettes, the genetic elements of RB47 binding site, any desired coding sequence, in combination with RB47 and RB60 coding sequences along with a promoter are readily combined in a number of operably linked permeations depending on the requirements of the cell system selected for the expression. For example, for expression in a chloroplast, endogenous RB47 protein is present therefore an expression cassette having an RB47 binding site and a desired coding sequence is minimally required along with an operative promoter sequence. Overexpression of RB47 may be preferable to enhance the translation of the coding sequence; in that case, the chloroplast is further transformed with an expression cassette containing an RB47-encoding sequence. Although the examples herein and below utilize primarily the sequence encoding the precursor form of RB47, any of the RB47-encoding sequences described in the present invention, i.e., RB47 precursor, mature RB47 and histidine-modified RB47 are contemplated for use in any expression cassette and system as described herein. To regulate the activation of translation, an RB60-encoding element is provided to the expression system to provide the ability to regulate redox potential in the cell as

AMENDED SHEET

taught in Section B. These examples herein and below represent a few of the possible permutations of genetic elements for expression in the methods of this invention.

In one embodiment, a plasmid is constructed containing an RB47 binding site directly upstream of an inserted coding region for a heterologous protein of interest, and the RB47 and RB60 coding regions. Heterologous refers to the nature of the coding region being dissimilar and not from the same gene as the regulatory molecules in the plasmid, such as RB47 and RB60. Thus, all the genetic elements of the present invention are produced in one transcript from the IPTG-inducible *psb*A promoter. Alternative promoters are similarly acceptable.

The final construct described herein for use in a prokaryotic expression system makes a single mRNA from which all three proteins are translated. The starting plasmid is any *E. coli* based plasmid containing an origin of replication and selectable marker gene. For this example, the Bluescript plasmid, pBS, commercially available through Stratagene, Inc., La Jolla, CA, which contains a polylinker-cloning site and an ampicilin resistant marker is selected for the vector.

The wild-type or native *psb*A gene (Erickson et al., Embo J., 3:2753-2762 (1984), also shown in Figures 3A-3C, is cloned into pBS at the EcoRI and BamHI sites of the polylinker. The nucleotide sequence of the *psb*A gene is available on GenBank with the 5' UTR and 3' UTR respectively listed in Accession Numbers X01424 and X02350. The EcoRI site of *psb*A is 1.5 kb upstream of

PCT/US 98/00840
IPEA/US 17 AUG 1998

the *psb*A initiation codon and the BamHI site is 2 kb downstream of the stop codon. This plasmid is referred to as pDl.

Using site-directed PCR mutagenesis, well known to one of ordinary skill in the art, an NdeI site is placed at the initiation codon of *psb*A in the pDl plasmid so that the ATG of the NdeI restriction site is the ATG initiation codon. This plasmid is referred to as pDl/Nde. An Nde site is then placed at the initiation codon of the gene encoding the heterologous protein of interest and an Xho I site is placed directly downstream (within 10 nucleotides) of the TAA stop codon of the heterologous protein coding sequence. Again using site-directed mutagenesis, an XhoI site is placed within 10 nucleotides of the initiation codon of RB47, the preparation of which is described in Example 2, and an NotI site is placed directly downstream of the stop codon of RB47. The heterologous coding region and the RB47 gene are then ligated into pDl/Nde so that the heterologous protein gene is directly adjacent to the RB47 binding site and the RB47 coding region is downstream of the heterologous coding region, using the Xho I site at the heterologous stop codon and the Not I site of the pDl polylinker.

These genetic manipulations result in a plasmid containing the 5' end of the *psb*A gene including the promoter region and with the RB47 binding site immediately upstream of a heterologous coding region, and the RB47 coding region immediately downstream of the heterologous coding region. The nucleotides between the stop codon of the heterologous coding region and the

AMENDED SHEET

PCT/US 98/00840
IPEA/US 17 AUG 1998

initiation codon of the RB47 coding region is preferably less than 20 nucleotides and preferably does not contain any additional stop codons in any reading frame. This plasmid is referred to as pD1/RB47.

Using site-directed mutagenesis, a NotI site is placed immediately (within 10 nucleotides) upstream of the initiation codon of RB60, the preparation of which is described in Example 2, and an Xba I site is placed downstream of the RB60 stop codon. This DNA fragment is then ligated to the 3' end of the *psb*A gene using the Xba I site found in the 3' end of the *psb*A gene so that the *psb*A 3' end is downstream of the RB60 coding region. This fragment is then ligated into the pD1/RB47 plasmid using the NotI and BamHI sites so that the RB60 coding region directly follows the RB47 coding region. The resulting plasmid is designated pD1/RB47/RB60. Preferably there is less then 20 nucleotides between the RB47 and RB60 coding regions and preferably there are no stop codons in any reading frame in that region. The final plasmid thus contains the following genetic elements operably linked in the 5' to 3' direction: the 5' end of the *psb*A gene with a promoter capable of directing transcription in chloroplasts, an RB47 binding site, a desired heterologous coding region, the RB47 coding region, the RB60 coding region, and the 3' end of the *psb*A gene which contains a transcription termination and mRNA processing site, and an *E. coli* origin of replication and amplicillin resistance gene. A diagram of this plasmid with the restriction sites is shown in Figure 4.

Expression of pD1/RB47/RB60 in *E. coli* to produce

PCT/US 98/00840
IPEA/US 17 AUG 1998

recombinant RB47, RB60 and the recombinant heterologous protein is performed as described in Example 4B. The heterologous protein is then purified as further described.

Expression cassettes in which the sequences encoding RB47 and RB60 are similarly operably linked to a heterologous coding sequence having the *psb*A RB47 binding site as described in Example 3 are prepared with a different promoter for use in eukaryotic, such as mammalian expression systems. In this aspect, the cassette is similarly prepared as described above with the exception that restriction cloning sites are dependent upon the available multiple cloning sites in the recipient vector. Thus, the RB47 binding site prepared in Example 3 is prepared for directed ligation into a selected expression vector downstream of the promoter in that vector. The RB47 and RB60 coding sequences are obtained from the pD1/RB47/RB60 plasmid by digestion with XhoI and XbaI and inserted into a similarly digested vector if the sites are present. Alternatively, site-directed mutagenesis is utilized to create appropriate linkers. A desired heterologous coding sequence is similarly ligated into the vector for expression.

B. Constructs Containing RB47 Nucleotide Sequence

1) Purified Recombinant RB47 Protein

In one approach to obtain purified recombinant RB47 protein, the full length RB47 cDNA prepared above was cloned into the *E. coli* expression vector pET3A (Studier et al., Methods Enzymol., 185:60-

PCT/US 98/00840
IPEA/US 17 AUG 1998

89 (1990)), also commercially available by Novagen, Inc., Madison, WI and transformed into BL21 *E. coli* cells. The cells were grown to a density of 0.4 (OD_{600}), then induced with 0.5 mM IPTG. Cells were then allowed to grow for an additional 4 hours, at which point they were pelleted and frozen.

Confirmation of the identity of the cloned cDNA as encoding the authentic RB47 protein was accomplished by examining protein expressed from the cDNA by immunoblot analysis and by RNA binding activity assay. The recombinant RB47 protein produced when the RB47 cDNA was expressed was recognized by antisera raised against the *C. reinhardtii* RB47 protein. The *E. coli* expressed protein migrated at 80 kDa on SDS-PAGE, but the protein was actually 69 kDa, as determined by mass spectrometry of the *E. coli* expressed protein. This mass agrees with the mass predicted from the cDNA sequence. A 60 kDa product was also produced in *E. coli,* and recognized by the antisera against the *C. reinhardtii* protein, which is most likely a degradation or early termination product of the RB47 cDNA. The recombinant RB47 protein expressed from the RB47 cDNA is recognized by the antisera raised against the *C. reinhardtii* protein at levels similar to the recognition of the authentic *C. reinhardtii* RB47 protein, demonstrating that the cloned cDNA produces a protein product that is immunologically related to the naturally produced RB47 protein. In order to generate a recombinant equivalent of the endogenous native RB47, the location of the 47 kDa polypeptide was mapped on the full-length recombinant protein by comparing mass spectrometric data of tryptic

PCT/US 98/00840
IPEA/US 17 AUG 1998

digests of the *C. reinhardtii* 47 kDa protein and the full-length recombinant protein. Thus, peptide mapping by mass spectrometry has shown that the endogenous RB47 protein corresponds primarily to the RNA binding domains contained within the N-terminal region of the predicted precursor protein, suggesting that a cleavage event is necessary to produce the mature 47 kDa protein. Thus, full-length recombinant RB47 is 69 kDa and contains a carboxy domain that is cleaved *in vivo* to generate the endogenous mature form of RB47 that is 47 kDa.

To determine if the heterologously expressed RB47 protein was capable of binding the *psb*A RNA, the *E. coli* expressed protein was purified by heparin agarose chromatography. The recombinant RB47 protein expressed in *E. coli* was purified using a protocol similar to that used previously for purification of RB47 from *C. reinhardtii*. Approximately 5 g of *E. coli* cells grown as described above were resuspended in low salt extraction buffer (10 mM Tris [pH 7.5], 10 mM NaCl, 10 mM $MgCl_2$, 5 mM ß-mercaptoethanol) and disrupted by sonication. The soluble cell extract was applied to a 5 mL Econo-Pac heparin cartridge (Bio-Rad) which was washed prior to elution of the RB47 protein (Danon and Mayfield, Embo J., 10:3993-4001 (1991)).

The *E. coli* expressed protein that bound to the heparin agarose matrix was eluted from the column at the same salt concentration as used to elute the authentic *C. reinhardtii* RB47 protein. This protein fraction was used in *in vitro* binding assays with the *psb*A 5' UTR. Both the 69 and 60 kDa *E. coli* expressed proteins crosslinked to the radiolabeled *psb*A 5' UTR at levels

similar to crosslinking of the endogenous RB47 protein, when the RNA/protein complex is subjected to UV irradiation.

Heparin agarose purified proteins, both from the *E. coli* expressed RB47 cDNA and from *C. reinhardtii* cells, were used in an RNA gel mobility shift assay to determine the relative affinity and specificity of these proteins for the 5' UTR of the *psb*A mRNA. The *E. coli* expressed proteins bound to the *psb*A 5' UTR *in vitro* with properties that are similar to those of the endogenous RB47 protein purified from *C. reinhardtii*. RNA binding to both the *E. coli* expressed and the endogenous RB47 protein was competed using either 200 fold excess of unlabeled *psb*A RNA or 200 fold excess of poly(A) RNA. RNA binding to either of these proteins was poorly competed using 200 fold excess of total RNA or 200 fold excess of the 5' UTR of the *psb*D or *psb*C RNAs. Different forms of the RB47 protein (47 kDa endogenous protein vs. the 69 kDa *E. coli* expressed protein) may account for the slight differences in mobility observed when comparing the binding profiles of purified *C. reinhardtii* protein to heterologously expressed RB47.

The mature form of RB47 is also produced in recombinant form by the insertion by PCR of an artificial stop codon in the RB47 cDNA at nucleotide positions 1403-1405 with a stop codon resulting in a mature RB47 recombinant protein having 402 amino acids as shown in Figures 1A-1D. An example of this is shown in Figures 5A-5B for the production of a recombinant histidine-modified RB47 mature protein as described

below. The complete RB47 cDNA is inserted into an expression vector, such as pET3A as described above, for expression of the mature 47 kDa form of the RB47 protein. In the absence of the inserted stop codon, the transcript reads through to nucleotide position 2066-2068 at the TAA stop codon to produce the precursor RB47 having the above-described molecular weight characteristics and 623 amino acid residues.

Recombinant RB47 is also expressed and purified in plant cells. For this aspect, *C. reinhardtii* strains were grown in complete media (Tris-acetate-phosphate [TAP] (Harris, The *Chlamydonas* Sourcebook, San Diego, CA, Academic Press (1989)) to a density of $5x10^6$ cells/mL under constant light. Cells were harvested by centrifugation at 4°C for 5 minutes at 4,000 *g*. Cells were either used immediately or frozen in liquid N_2 for storage at -70°C.

Recombinant RB47 protein was also produced as a modified RB47 protein with a histidine tag at the amino-terminus according to well known expression methods using pET19-D vectors available from Novagen, Inc., Madison, WI. The nucleotide and amino acid sequence of a recombinant histidine-modified RB47 of the mature 47 kDa form is shown in Figures 5A-5B with the nucleotide and amino acid sequence also listed in SEQ ID NO 14. Thus the nucleotide sequence of a histidine-modified RB47 is 1269 bases in length. The precursor form of the RB47 protein is similarly obtained in the expression system, both of which are modified by the presence of a histidine tag that allows for purification by metal affinity chromatography.

PCT/US 98/00840
IPEA/US 17 AUG 1998

The recombinant histidine-modified RB47 purified through addition of a poly-histidine tag followed by Ni^{+2} column chromatography showed similar binding characteristics as that described for recombinant precursor RB47 described above.

C. Constructs Containing RB60 Nucleotide Sequence

In one approach to obtain purified recombinant RB60 protein, the full-length RB60 cDNA prepared above was cloned into the *E. coli* expression vector pET3A (Studier et al., Methods Enzymol., 185:60-89 (1990)), also commercially available by Novagen, Inc., Madison, WI and transformed into BL21 *E. coli* cells. The cells were grown to a density of 0.4 (OD_{600}), then induced with 0.5 mM IPTG. Cells were then allowed to grow for an additional 4 hours, at which point they were pelleted and frozen.

Recombinant histidine-modified RB60 was also expressed with a pET19-D vector as described above for RB47 that was similarly modified. Purification of the recombinant RB60 proteins was performed as described for RB47 thereby producing recombinant RB60 proteins for use in the present invention.

The RB60 coding sequence is also mutagenized for directional ligation into an selected vector for expression in alternative systems, such as mammalian expression systems.

D. Constructs Containing an RB47-Encoding Sequence and an RB60-Encoding Sequence

To prepare an expression cassette for encoding

PCT/US 98/00840

IPEA/US 17 AUG 1998

both RB47 and RB60, one approach is to digest plasmid pD1/RB47/RB60 prepared above with XhoI and XbaI to isolate the fragment for both encoding sequences. The fragment is then inserted into a similarly digested expression vector if available or is further mutagenized to prepare appropriate restriction sites.

Alternatively, the nucleotide sequences of RB47 and RB60, as described in Example 2, are separately prepared for directional ligation into a selected vector.

An additional embodiment of the present invention is to prepare an expression cassette containing the RB47 binding site along with the coding sequences for RB47 and RB60, the plasmid pD1/RB47/RB60 prepared above is digested with NdeI and XhoI to prepare an expression cassette in which any desired coding sequence having similarly restriction sites is directionally ligated. Expression vectors containing both the RB47 and RB60 encoding sequences in which the RB47 binding site sequence is utilized with a different promoter are also prepared as described in Example 4A.

E. <u>Constructs Containing an RB47 Binding Site Nucleotide Sequence, Insertion Sites for a Desired Heterologous Coding Sequence, and an RB47-Encoding Sequence</u>

In another permutation, a plasmid or expression cassette is constructed containing an RB47 binding site directly upstream of an inserted coding region for a heterologous protein of interest, and the RB47 coding region. The final construct described herein for use in a prokaryotic expression system makes

a single mRNA from which both proteins are translated.

The plasmid referred to as pD1/RB47 is prepared as described above in Example 4A. A diagram of this plasmid with the restriction sites is shown in Figure 6.

Expression of pD1/RB47 in *E. coli* to produce recombinant RB47 and the recombinant heterologous protein is performed as described in above. The heterologous protein is then purified as further described.

To produce an expression cassette that allows for insertion of an alternative desired coding sequence, the plasmid pD1/RB47 is digested with NdeI and XhoI resulting in a vector having restriction endonuclease sites for insertion of a desired coding sequence operably linked to a RB47 binding site and RB47 coding sequence on one transcriptional unit.

F. <u>Constructs Containing an RB47 Binding Site Nucleotide Sequence, Insertion Sites for a Desired Heterologous Coding Sequence, and an RB47-Encoding Sequence</u>

In another permutation, a plasmid or expression cassette is constructed containing an RB47 binding site directly upstream of an inserted coding region for a heterologous protein of interest, and the RB60 coding region. The final construct described herein for use in a prokaryotic expression system makes a single mRNA from which both proteins are translated. In this embodiment, a separate construct encoding recombinant RB47 as described in Example 4B is co-transformed into the *E. coli* host cell for expression.

The plasmid referred to as pD1/RB60 is prepared as described above for pD1/RB47 in Example 4A with the exception that XhoI and XbaI sites are created on RB60 rather than RB47.

Expression of pD1/RB60 in *E. coli* to produce recombinant RB60 and the recombinant heterologous protein is performed as described in above with the combined expression of RB47 from a separate expression cassette. The heterologous protein is then purified as further described.

To produce an expression cassette that allows for insertion of an alternative desired coding sequence, the plasmid pD1/RB60 is digested with NdeI and XhoI resulting in a vector having restriction endonuclease sites for insertion of a desired coding sequence operably linked to a RB47 binding site and RB60 coding sequence on one transcriptional unit.

G. <u>Constructs Containing RB47 Binding Site Nucleotide Sequence and Heterologous Coding Sequences</u>

1) <u>Expression of Recombinant Tetanus Toxin Single Chain Antibody</u>

The examples herein describe constructs that are variations of those described above. The constructs described below contain an RB47 binding site sequence and a heterologous coding sequence. The activating protein RB47 was endogenously provided in the chloroplast and or plant cell. In other aspects however as taught by the methods of the present invention, the chloroplast is further transformed with an RB47-

expression construct as described above for overexpression of RB47 to enhance translation capacities.

A strain of the green algae *Chlamydomonas reinhardtii* was designed to allow expression of a single chain antibody gene in the chloroplast. The transgenically expressed antibody was produced from a chimeric gene containing the promoter and 5' untranslated region (UTR) of the chloroplast *psb*A gene prepared as described above, followed by the coding region of a single chain antibody (encoding a tetanus toxin binding antibody), and then the 3' UTR of the *psb*A gene also prepared as described above to provide for transcription termination and RNA processing signals. This construct is essentially pD1/Nde including a heterologous coding sequence having a 3' XbaI restriction site for ligation with the 3' *psb*A gene and is diagramed in Figure 7.

The *psb*A-single chain construct was first transformed into *C. reinhardtii* chloroplast and transformants were then screened for single chain gene integration. Transformation of chloroplast was performed via bolistic delivery as described in US Patents 5,545,818 and 5,553,878, the disclosures of which are hereby incorporated by reference. Transformation is accomplished by homologous recombination via the 5' and 3' UTR of the *psb*A mRNA.

As shown in Figure 8, two of the transformants that contained the single chain chimeric gene produced single chain antibodies at approximately 1% of total protein levels. The transgenic antibodies were of the correct

PCT/US 98/00840
IPEA/US 17 AUG 1998

size and were completely soluble, as would be expected of a correctly folded protein. Few degradation products were detectable by this Western analysis, suggesting that the proteins were fairly stable within the chloroplast. To identify if the produced antibody retained the binding capacity for tetanus toxin, ELISA assays were performed using a mouse-produced Fab, from the original tetanus toxin antibody, as the control. The chloroplast single chain antibody bound tetanus toxin at levels similar to Fab, indicating that the single chain antibody produced in *C. reinhardtii* is a fully functional antibody. These results clearly demonstrate the ability of the chloroplast to synthesis and accumulate function antibody molecules resulting from the translational activation of an RB47 binding site in an expression cassette by endogenous RB47 protein in the chloroplast.

2) Expression of Bacterial Luciferase Enzyme Having Two Subunits

For the production of molecules that contain more than one subunit, such as dIgA and bacterial luciferase enzyme, several proteins must be produced in stoichiometric quantities within the chloroplast. Chloroplast have an advantage for this type of production over cytoplasmic protein synthesis in that translation of multiple proteins can originate from a single mRNA. For example, a dicistronic mRNA having 5' and 3' NdeI and XbaI restriction sites and containing both the A and B chains of the bacterial luciferase enzyme was inserted downstream of the *psbA* promoter and

5' UTR of the pD1/Nde construct prepared in Example 4A above. In this construct, the bacterial LuxAB coding region was ligated between the *psb*A 5' UTR and the *psb*A 3' end in an *E. coli* plasmid that was then transformed into *Chlamydomonas reinhardtii* cells as described above for expression in the chloroplast. A schematic of the construct is shown in Figure 9. Single transformant colonies were then isolated. A plate containing a single isolate was grown for 10 days on complete media and a drop of the luciferase substrate n-Decyl Aldehyde was placed on the plate and the luciferase visualized by video-photography in a dark chamber. Both proteins were synthesized from this single mRNA and luciferase activity accumulated within the chloroplast as shown in Figure 10. Some mRNA within plastids contained as many as 5 separate proteins encoded on a single mRNA.

3) <u>Expression of Dimeric IgA</u>

To generate dimeric IgA, the construct shown in Figure 11 is engineered so that the *psb*A promoter and 5' UTR are used to drive the synthesis of the light chain and heavy chains of an antibody, and the J chain normally associated with IgA molecules. The nucleic acid sequences for the dimeric IgA are inserted into the RB47 binding site construct prepared in Example 4A. The construct is then transformed into *C. reinhardtii* cells as previously described for expression of the recombinant dIgA.

Production of these three proteins within the plastid allows for the self assembly of a dimeric IgA (dIgA). Production of this complex is monitored in

PCT/US 98/00840
IPEA/US 17 AUG 1998

several ways. First, Southern analysis of transgenic algae is used to identify strains containing the polycistronic chimeric dIgA gene. Strains positive for integration of the dIgA gene are screened by Northern analysis to ensure that the chimeric mRNA is accumulating. Western blot analysis using denaturing gels is used to monitor the accumulation of the individual light, heavy and J chain proteins, and native gels Western blot analysis will be used to monitor the accumulation of the assembled dIgA molecule.

By using a single polycistronic mRNA in the context of RB47 regulated translation, two of the potential pitfalls in the assembly of multimeric dIgA molecule are overcome. First, this construct ensures approximately stoichiometric synthesis of the subunits, as ribosomes reading through the first protein are likely to continue to read through the second and third proteins as well. Second, all of the subunits are synthesized in close physical proximity to each other, which increases the probability of the proteins self assembling into a multimeric molecule. Following the production of a strain producing dIgA molecules, the production of dIgA on an intermediate scale by growing algae in 300 liter fermentors is then performed. Larger production scales are then performed thereafter.

The foregoing specification, including the specific embodiments and examples, is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the invention.

PCT/US 98/00840
IPEA/US 17 AUG 1998

What Is Claimed Is:

1. An expression cassette for expression of a desired molecule, which cassette comprises:

a) an RB47 binding site nucleotide sequence upstream of a restriction endonuclease site for insertion of a desired coding sequence to be expressed; and

b) a nucleotide sequence encoding a polypeptide which binds RB47 binding site.

2. The expression cassette of claim 1 further comprising a promoter sequence operably linked to and positioned upstream of the RB47 binding site nucleotide sequence.

3. The expression cassette of claim 2 wherein the promoter sequence is derived from a *psbA* gene.

4. The expression cassette of claim 3 wherein the coding sequence is heterologous to the *psbA* gene.

5. The expression cassette of claim 1 wherein the cassette comprises a plasmid or virus.

6. The expression cassette of claim 1 further comprising and operably linked thereto a nucleotide sequence encoding RB60.

7. The expression cassette of claim 1 wherein the RB47 binding polypeptide is selected from the group consisting of RB47, RB47 precursor and a histidine-modified RB47.

8. An expression cassette for expression of a desired molecule, which cassette comprises:

a) an RB47 binding site nucleotide sequence upstream of a restriction endonuclease site for insertion of a desired coding sequence to be expressed;

PCT/US 98/00840
IPEA/US 17 AUG 1998

and

b) a nucleotide sequence encoding a polypeptide which regulates the binding of RB47 to the RB47 binding site.

9. The expression cassette of claim 8 wherein the regulatory polypeptide is RB60.

10. A recombinant RB47 protein.

11. A recombinant RB60 protein.

12. An isolated nucleotide sequence encoding RB47.

13. An isolated nucleotide sequence encoding a histidine-modified RB47.

14. An isolated nucleotide sequence encoding RB47 precursor.

15. The nucleotide sequence of claim 12 from nucleotide position 197 to 1402 in Figures 1A-1B and SEQ ID NO 5.

16. The nucleotide sequence of claim 13 from nucleotide position 1 to 1269 in Figures 5A-5B and SEQ ID NO 14.

17. The nucleotide sequence of claim 14 shown in from nucleotide position 197 to 2065 in Figures 1A-1C and SEQ ID NO 5.

18. An expression cassette comprising the nucleotide sequence of claim 12, 13 or 14.

19. An isolated nucleotide sequence encoding RB60.

20. The nucleotide sequence of claim 18 from nucleotide position 16 to 1614 in Figures 2A-2B and SEQ ID NO 10.

21. An expression cassette comprising the nucleotide sequence of claim 19.

22. An expression system comprising a cell

PCT/US 98/00840
IPEA/US 17 AUG 1998

transformed with the expression cassette of claim 1.

23. The expression system of claim 22 wherein the cell is a plant cell.

24. The expression system of claim 23 wherein the plant cell endogenously expresses RB47.

25. The expression system of claim 23 wherein the plant cell endogenously expresses RB60.

26. The expression system of claim 23 wherein the plant cell endogenously expresses RB47 and RB60.

27. The expression system of claim 22 wherein the cell is a eukaryotic cell.

28. The expression system of claim 22 wherein the cell is a prokaryotic cell.

29. The expression system of claim 22 further comprising the expression cassette of claim 21.

30. An expression system comprising a cell transformed with the expression cassette of claim 8.

31. The expression system of claim 29 further comprising the expression cassette of claim 18.

32. A cell stably transformed with the expression cassette of claim 18.

33. A cell stably transformed with the expression cassette of claim 21.

34. A cell stably transformed with the expression cassette of claims 18 and 21.

35. The expression cassette of claim 1 further comprising an inserted desired coding sequence.

36. An expression system comprising a cell transformed with the expression cassette of claim 35, wherein the coding sequence is expressed forming the desired molecule upon activation of the RB47 binding

PCT/US 98/00840
IPEA/US 17 AUG 1998

site with RB47.

37. The expression system of claim 36 wherein the cell is a plant cell endogenously expressing RB47.

38. The expression system of claim 36 wherein the cell is stably transformed with the expression cassette of claim 21.

39. An expression system comprising a cell transformed with an expression cassette comprising a promoter sequence, a RB47 binding site sequence, a desired coding sequence for a molecule, and a nucleotide sequence for encoding a polypeptide which binds RB47 binding site, wherein all sequences are operably linked.

40. A method of preparing a desired recombinant molecule wherein the method comprises cultivating the expression system of claim 36.

41. A method of preparing a desired recombinant molecule wherein the method comprises cultivating the expression system of claim 39.

42. A method for expressing a desired coding sequence comprising:

a) forming an expression cassette by operably linking:

1) a promoter sequence;

2) a RB47 binding site sequence;

3) a desired coding sequence; and

4) a nucleotide sequence encoding a polypeptide which binds RB47 binding site; and

b) introducing the expression cassette into a cell.

43. The method of claim 42 wherein the cell is a plant cell endogenously expressing RB47.

PCT/US 98/00840
IPEA/US 17 AUG 1998

44. The method of claim 42 wherein the cell is a plant cell endogenously expressing RB60.

45. The method of claim 42 further comprising inducing expression with a promoter inducer molecule.

46. The method of claim 45 wherein the promoter inducer molecule is IPTG.

47. The method of claim 42 wherein the cell is transformed with the expression cassette of claim 21.

48. A method for expressing a desired coding sequence comprising:

a) forming an expression cassette by operably linking:

1) a promoter sequence;

2) a RB47 binding site sequence; and

3) a desired coding sequence;

and

b) introducing the expression cassette into a plant cell endogenously expressing RB47.

49. The method of claim 48 wherein the expression cassette further comprises a nucleotide sequence encoding RB60.

50. A method for the regulated production of a recombinant molecule from a desired coding sequence in a cell, wherein the cell contains the expression cassette of claim 34, wherein expression of the coding sequence is activated by RB47 binding to the RB47 binding site thereby producing the recombinant molecule.

51. A method of forming an expression cassette by operably linking:

a) a RB47 binding site sequence;

b) a cloning site for insertion of a desired

PCT/US 98/00840
IPEA/US 17 AUG 1998

coding sequence downstream of the RB47 binding site sequence; and

c) a nucleotide sequence encoding a polypeptide which binds the RB47 binding site.

52. The method of claim 51 further comprising a promoter sequence operably linked upstream to the RB47 binding site sequence.

53. The method of claim 51 further comprising a desired coding sequence inserted into the insertion site.

54. A method of screening for agonists or antagonists of RB47 binding to RB47 binding site, the method comprising the steps:

a) providing a cell expression system containing:

1) a promoter sequence;

2) a RB47 binding site sequence;

3) a coding sequence for an indicator polypeptide; and

4) a polypeptide which binds to the RB47 binding site sequence;

b) introducing an antagonist or agonist into the cell; and

c) detecting the amount of indicator polypeptide expressed in the cell.

55. A method of screening for agonists or antagonists of RB60 in regulating RB47 binding to RB47 binding site, the method comprising the steps:

a) providing an expression system in a cell containing:

1) a promoter sequence;

PCT/US 98/00840
IPEA/US 17 AUG 1998

2) a RB47 binding site sequence;

3) a coding sequence for an indicator polypeptide;

4) a polypeptide which binds to the RB47 binding site sequence; and

5) a RB60 polypeptide;

b) introducing an agonist or antagonist into the cell; and

c) detecting the amount of indicator polypeptide expressed in the cell.

56. An article of manufacture comprising a packaging material and contained therein in a separate container the expression cassette of claim 1, wherein the expression cassette is useful for expression of a desired coding sequence, and wherein the packaging material comprises a label which indicates that the expression cassette can be used for expressing a desired coding sequence when the RB47 binding site is activated by RB47.

57. The article of manufacture of claim 56 further comprising in a separate container the expression cassette of claim 18.

58. The article of manufacture of claim 56 further comprising in a separate container the expression cassette of claim 21.

59. An article of manufacture comprising a packaging material and contained therein in a separate container the expression system of claim 22, wherein the expression system is useful for expression of a desired coding sequence, and wherein the packaging material comprises a label which indicates that the expression

PCT/US 98/00840
IPEA/US 17 AUG 1998

system can be used for expressing a desired coding sequence when the RB47 binding site is activated by RB47.

60. An article of manufacture comprising a packaging material and contained therein in a separate container the stably transformed cell of claim 32, wherein the cell is useful as an expression system, and wherein the packaging material comprises a label which indicates that the expression system can be used for expressing a desired coding sequence when the RB47 binding site is activated by RB47.

61. An article of manufacture comprising a packaging material and contained therein in a separate container the stably transformed cell of claim 33, wherein the cell is useful as an expression system, and wherein the packaging material comprises a label which indicates that the expression system can be used for expressing a desired coding sequence when the RB47 binding site is activated by RB47 and regulated by RB60.

62. An article of manufacture comprising a packaging material and contained therein in a separate container the stably transformed cell of claim 34, wherein the cell is useful as an expression system, and wherein the packaging material comprises a label which indicates that the expression system can be used for expressing a desired coding sequence when the RB47 binding site is activated by RB47 and regulated by RB60.

63. An article of manufacture comprising a packaging material and contained therein in a separate container the expression cassette of claim 2, wherein the expression cassette is useful for expression of a

RNA transcript, and wherein the packaging material comprises a label which indicates that the expression cassette can be used for producing *in vitro* a RNA transcript when the RB47 binding site is activated by RB47.

64. The article of manufacture of claim 63 wherein the promoter sequence is selected from the group consisting of T3 and T7 promoters.

65. The article of manufacture of claim 63 further comprising in separate containers a polymerase, a buffer and each of four ribonucleotides, reagents for *in vitro* RNA transcription.

```
  1 GAATTCGCGGCCGCTCCGTGGTTGGTCCTC ATG GTG TCT TTT TGA AGAGGACCTGAGCCTTTCACCCAAATATA 74
  1                                M   V   S   F   *                                 5

 75 TCAAAAAACCCGGGCAACCGGCCAAAAAAATTGCAAAAGCCTCTCGTAGGCACAAAAGACCTATTCTAGCCATCAACTTT 154

155 GTATCCGACGCTGCCGTTTAGCTGCGCGTCTTGAAGTCAAGC ATG GCG ACT ACT GAG TCC TCG GCC CCG   223
  1                                            M   A   T   T   E   S   S   A   P     9

224 GCG GCC ACC ACC CAG CCG GCC AGC ACC CCG CTG GCG AAC TCG TCG CTG TAC GTC GGT GAC 283
 10 A   A   T   T   Q   P   A   S   T   P   L   A   N   S   S   L   Y   V   G   D    29

284 CTG GAG AAG GAT GTC ACC GAG GCC CAG CTG TTC GAG CTC TTC TCC TCG GTT GGC CCT GTG 343
 30 L   E   K   D   V   T   E   A   Q   L   F   E   L   F   S   S   V   G   P   V    49

344 GCC TCC ATT CGC GTG TGC CGC GAT GCC GTC ACG CGC CGC TCG CTG GGC TAC GCC TAC GTC 403
 50 A   S   I   R   V   C   R   D   A   V   T   R   R   S   L   G   Y   A   Y   V    69

404 AAC TAC AAC AGC GCT CTG GAC CCC CAG GCT GCT GAC CGC GCC ATG GAG ACC CTG AAC TAC 463
 70 N   Y   N   S   A   L   D   P   Q   A   A   D   R   A   M   E   T   L   N   Y    89

464 CAT GTC GTG AAC GGC AAG CCT ATG CGC ATC ATG TGG TCG CAC CGC GAC CCT TCG GCC CGC 523
 90 H   V   V   N   G   K   P   M   R   I   M   W   S   H   R   D   P   S   A   R    109

524 AAG TCG GGC GTC GGC AAC ATC TTC ATC AAG AAC CTG GAC AAG ACC ATC GAC GCC AAG GCC 583
110 K   S   G   V   G   N   I   F   I   K   N   L   D   K   T   I   D   A   K   A    129

584 CTG CAC GAC ACC TTC TCG GCC TTC GGC AAG ATT CTG TCC TGC AAG GTT GCC ACT GAC GCC 643
130 L   H   D   T   F   S   A   F   G   K   I   L   S   C   K   V   A   T   D   A    149

644 AAC GGC GTG TCG AAG GGC TAC GGC TTC GTG CAC TTC GAG GAC CAG GCC GCT GCC GAT CGC 703
150 N   G   V   S   K   G   Y   G   F   V   H   F   E   D   Q   A   A   A   D   R    169

704 GCC ATT CAG ACC GTC AAC CAG AAG AAG ATT GAG GGC AAG ATC GTG TAC GTG GCC CCC TTC 763
170 A   I   Q   T   V   N   Q   K   K   I   E   G   K   I   V   Y   V   A   P   F    189
```

FIG. 1A

```
764  CAG AAG CGC GCT GAC CGC CCC AGG GCA AGG ACG TTG TAC ACC AAC GTG TTC GTC AAG AAC  823
190  Q   K   R   A   D   R   P   R   A   R   T   L   Y   T   N   V   F   V   K   N    209

824  TTG CCG GCC GAC ATC GGC GAC GAC GAG CTG GGC AAG ATG GCC ACC GAG CAC GGC GAG ATC  883
210  L   P   A   D   I   G   D   D   E   L   G   K   M   A   T   E   H   G   E   I    229

884  ACC AGC GCG GTG GTC ATG AAG GAC GAC AAG GGC GGC AGC AAG GGC TTC GGC TTC ATC AAC  943
230  T   S   A   V   V   M   K   D   D   K   G   G   S   K   G   F   G   F   I   N    249

944  TTC AAG GAC GCC GAG TCG GCG GCC AAG TGC GTG GAG TAC CTG AAC GAG CGC GAG ATG AGC  1003
250  F   K   D   A   E   S   A   A   K   C   V   E   Y   L   N   E   R   E   M   S    269

1004 GGC AAG ACC CTG TAC GCC GGC CGC GCC CAG AAG AAG ACC GAG CGC GAG GCG ATG CTG CGC  1063
270  G   K   T   L   Y   A   G   R   A   Q   K   K   T   E   R   E   A   M   L   R    289

1064 CAG AAG GCC GAG GAG AGC AAG CAG GAG CGT TAC CTG AAG TAC CAG AGC ATG AAC CTG TAC  1123
290  Q   K   A   E   E   S   K   Q   E   R   Y   L   K   Y   Q   S   M   N   L   Y    309

1124 GTC AAG AAC CTG TCC GAC GAG GAG GTC GAC GAC GAC GCC CTG CGT GAG CTG TTC GCC AAC  1183
310  V   K   N   L   S   D   E   E   V   D   D   D   A   L   R   E   L   F   A   N    329

1184 TCT GGC ACC ATC ACC TCG TGC AAG GTC ATG AAG GAC GGC AGC GGC AAG TCC AAG GGC TTC  1243
330  S   G   T   I   T   S   C   K   V   M   K   D   G   S   G   K   S   K   G   F    349

1244 GGC TTC GTG TGC TTC ACC AGC CAC GAC GAG GCC ACC CGG CCG CCC GTG ACC GAG ATG AAC  1303
350  G   F   V   C   F   T   S   H   D   E   A   T   R   P   P   V   T   E   M   N    369

1304 GGC AAG ATG GTC AAG GGC AAG CCC CTG TAC GTG GCC CTG GCG CAG CGC AAG GAC GTG CGC  1363
370  G   K   M   V   K   G   K   P   L   Y   V   A   L   A   Q   R   K   D   V   R    389

1364 CGT GCC ACC CAG CTG GAG GCC AAC ATG CAG GCG CGC ATG GGC ATG GGC GCC ATG AGC CGC  1423
390  R   A   T   Q   L   E   A   N   M   Q   A   R   M   G   M   G   A   M   S   R    409
```

FIG. 1B

```
1424 CCG CCG AAC CCG ATG GCC GGC ATG AGC CCC TAC CCC GGC GCC ATG CCG TTC TTC GCT CCC 1483
 410 P   P   N   P   M   A   G   M   S   P   Y   P   G   A   M   P   F   F   A   P   429

1484 GGC CCC GGC GGC ATG GCT GCT GGC CCG CGC GCT CCG GGC ATG ATG TAC CCG CCC ATG ATG 1543
 430 G   P   G   G   M   A   A   G   P   R   A   P   G   M   M   Y   P   P   M   M   449

1544 CCG CCG CGC GGC ATG CCT GGC CCC GGC CGC GGC CCC CGC GGC CCC ATG ATG CCG CCC CAG 1603
 450 P   P   R   G   M   P   G   P   G   R   G   P   R   G   P   M   M   P   P   Q   469

1604 ATG ATG GGT GGC CCC ATG ATG GGC CCG CCC ATG GGC CCC GGG CGC GGC CGT GGC GGC CGC 1663
 470 M   M   G   G   P   M   M   G   P   P   M   G   P   G   R   G   R   G   G   R   489

1664 GGC CCC TCC GGC CGC GGC CAG GGC CGC GGC AAC AAC GCC CCT GCC CAG CAG CCC AAG CCC 1723
 490 G   P   S   G   R   G   Q   G   R   G   N   N   A   P   A   Q   Q   P   K   P   509

1724 GCC GCT GAG CCG GCC GCC GCG CCC GCC GCC GCC GCC CCC GCT GCC GCG GCG CCT GCC GCC 1783
 510 A   A   E   P   A   A   A   P   A   A   A   A   P   A   A   A   A   P   A   A   529

1784 GCG GCG GAG CCG GAG GCC CCC GCC GCC CAG CAG CCG CTG ACC GCC TCC GCG CTG GCC GCC 1843
 530 A   A   E   P   E   A   P   A   A   Q   Q   P   L   T   A   S   A   L   A   A   549

1844 GCC GCG CCG GAG CAG CAG AAG ATG ATG ATC GGC GAG CGC CTG TAC CCG CAG GTG GCG GAG 1903
 550 A   A   P   E   Q   Q   K   M   M   I   G   E   R   L   Y   P   Q   V   A   E   569

1904 CTG CAG CCC GAC CTG GCT GGC AAG ATC ACC GGC ATG CTG CTG GAG ATG GAC AAC GCC GAG 1963
 570 L   Q   P   D   L   A   G   K   I   T   G   M   L   L   E   M   D   N   A   E   589

1964 CTT CTG ATG CTT CTG GAG TCG CAC GAG GCG CTG GTG TCC AAG GTG GAC GAG GCC ATC GCT 2023
 590 L   L   M   L   L   E   S   H   E   A   L   V   S   K   V   D   E   A   I   A   609

2024 GTG CTC AAG CAG CAC AAC GTG ATT GCC GAG GAG AAC AAG GCT TAA AGCGCCTGCACGCTTGTGCG 2088
 610 V   L   K   Q   H   N   V   I   A   E   E   N   K   A   *                        624
```

FIG. 1C

```
2089 GGCTGGTGGCGCCGGCGCGCGCCGGCGCTGCTTGGGCCGCCGGCAGC ATG GGC GCG GCG GAC GCG GTG TGG 2159
   1                                               M   G   A   A   D   A   V   W   8

2160 GAG CAG TGC TTG CTG CTT CTG GCC GCC GTG AAG CCG CGC CGA ACT GGG GCG GAC GGC AGG 2219
   9 E   Q   C   L   L   L   L   A   A   V   K   P   R   R   T   G   A   D   G   R   28

2220 CTG GCG TTG ACG CCG GCG CGC CAC AAC ACA AAG TTG GTG GCG TGA AAGTCTCTGGGCGTGCTCCG 2284
  29 L   A   L   T   P   A   R   H   N   T   K   L   V   A   *                        43

2285 GACGGTTGTAAGGTTTTAAGAACTGGCTTTTGGCCGGGTTGCCGCCCAAAGGCGGAACGGCGGTCTTTTCAGGCCAATCA 2364

2365 CATCCGGCTGGAAAAATTCTTACCAAAGCCAACCCCTGCACCCAAAAATTTCGGGTTCCGAAAGAACACTCCCCTTTTTT 2444

2445 CCGGCAACGCGTTCTTTCAAGGCCAATCACTTTCCGGGTTGGAAGAAA ATG TTA CCC GGA AAA GGC GGG AAG 2516
   1                                                M   L   P   G   K   G   G   K   8

2517 CCC CCT GCA CCC GGA CAA GTT ATT CGG GGT TTC GCC GGG AAT GAG CAA GCG TTC GGG CTG 2576
   9 P   P   A   P   G   Q   V   I   R   G   F   A   G   N   E   Q   A   F   G   L   28

2577 TTG GCC GTA TCG CGA ACG CTG TCG GGG TGT CAG GCG CCA GAA GGA AGG ATG ACG TTT TGG 2636
  29 L   A   V   S   R   T   L   S   G   C   Q   A   P   E   G   R   M   T   F   W   48

2637 TGA AGGGGTGCAAACTGAGCACACGAGTTTTGGCAATAGACGTGGAGAAAGTCCAGTGCGGGGTGAGGCGGATAGCGGA 2715
  49 *                                                                                49

2716 ATCAAGCGTGGCGGGTCCCTGGCGAGACGAGACGCTTCTGTTGTTTTGCTGAGCCCTTTG ATG GCA CAA TCG CAC 2790
   1                                                            M   A   Q   S   H   5

2791 TGT TTT GAG CAG GCG ACT GTA AAG TGC CCG ACG CTA AAA AAG CGG CCG CGA ATT CC      2846
   6 C   F   E   Q   A   T   V   K   C   P   T   L   K   K   R   P   R   I           23
```

FIG. 1D

RECTIFIED SHEET (RULE 91)

MNRWNLLALTLGLLLVAAPFTKHQFAHASDEYEDDEEDDAPAAP

KDDDVDVTVVTVKNWDETVKKSKFALVEFYAPWCGHCKTLKPEYAKAATALKAAAPDA

LIAKVDATQEESLAQKFGVQGYPTLKWFVDGELASDYNGPRDADGIVGWVKKKTGPPA

VTVEDADKLKSLEADAEVVVVGYFKALEGEIYDTFKSYAAKTEDVVFVQTTSADVAKA

AGLDAVDTVSVVKNFAGEDRATAVLATDIDTDSLTAFVKSEKMPPTIEFNQKNSDKIF

NSGINKQLILWTTADDLKADAEIMTVFREASKKFKGQLVFVTVNNEGDGADPVTNFFG

LKGATSPVLLGFFMEKNKKFRMEGEFTADNVAKFAESVVDGTAQAVLKSEAIPEDPYE

DGVYKIVGKTVESVVLDETKDVLLEVYAPWCGHCKKLEPIYKKLAKRFKKVDSVIIAK

MDGTENEHPEIEVKGFPTILFYPAGSDRTPIVFEGGDRSLKSLTKFIKTNAKIPYELP
KKGSDGDEGTSDDKDKPASDKDEL

```
  1 gagtacgttt acgccatgaa ccgttggaac cttcttgccc ttaccctggg gctgctgctg
 61 gtggcagcgc ccttcaccaa gcaccagttt gctcatgctt ccgatgagta tgaggacgac
121 gaggaggacg atgcccccgc cgcccctaag gacgacgacg tcgacgttac tgtggtgacc
181 gtcaagaact gggatgagac cgtcaagaag tccaagttcg cgcttgtgga gttctacgct
241 ccttggtgcg gccactgcaa gaccctcaag cctgagtacg ctaaggctgc caccgccctg
301 aaggctgctg ctcccgatgc ccttatcgcc aaggtcgacg ccacccagga ggagtccctg
361 gcccagaagt tcggcgtgca gggctacccc accctcaagt ggttcgttga tggcgagctg
421 gcttctgact acaacggccc ccgcgacgct gatggcattg ttggctgggt gaagaagaag
481 actggccccc ccgccgtgac cgttgaggac gccgacaagc tgaagtccct ggaggcggac
541 gctgaggtcg ttgtcgtcgg ctacttcaag gccctggagg gcgagatcta cgacaccttc
601 aagtcctacg ccgccaagac cgaggacgtg gtgttcgtgc agaccaccag cgccgacgtc
```

FIG. 2A

661 gccaaggccg ccggcctgga cgccgtggac accgtgtccg tggtcaagaa cttcgccggt
721 gaggaccgcg ccaccgccgt cctggccacg gacatcgaca ctgactccct gaccgcgttc
781 gtcaagtcgg agaagatgcc ccccaccatt gagttcaacc agaagaactc tgacaagatc
841 ttcaacagcg gcatcaacaa gcagctgatt ctgtggacca ccgccgacga cctgaaggcc
901 gacgccgaga tcatgactgt gttccgcgag gccagcaaga agttcaaggg ccagctggtg
961 ttcgtgaccg tcaacaacga gggcgacggc gccgaccccg tcaccaactt cttcggcctc
1021 aagggcgcca cctcgcctgt gctgctgggc ttcttcatgg agaagaacaa gaagttccgc
1081 atggagggcg agttcacggc tgacaacgtg gctaagttcg ccgagagcgt ggtggacggc
1141 accgcgcagg ccgtgctcaa gtcggaggcc atccccgagg acccctatga ggatggcgtc
1201 tacaagattg tgggcaagac cgtggagtct gtggttctgg acgagaccaa ggacgtgctg
1261 ctggaggtgt acgccccctg gtgcggccac tgcaagaagc tggagcccat ctacaagaag
1321 ctggccaagc gctttaagaa ggtggattcc gtcatcatcg ccaagatgga tggcactgag
1381 aacgagcacc ccgagatcga ggtcaagggc ttccctacca tcctgttcta tcccgccggc
1441 agcgaccgca cccccatcgt gttcgagggc ggcgaccgct cgctcaagtc cctgaccaag
1501 ttcatcaaga ccaacgccaa gatcccgtac gagctgccca agaagggctc cgacggcgac
1561 gagggcacct cggacgacaa ggacaagccc gcgtccgaca aggacgagct gtaagcggct
1621 atctgaacta ccccaggttt ggagcgtctg cttgcgcgct tgcgcttgca cactgtgcat
1681 ggatgggagt taaggaggag acggagcacg gaggctgcgc tcggttggtg gcttggagca
1741 ccggcagcgc gtgatccgtc ctggcagcag caacggcgga gcgggcgcat attggcgcga
1801 gctggcgagc ggctgttgct ggagaggata tgctgccggg cgggaggaag ggctaggggc
1861 agagatgaga gcgttacggg ctggcatgcg ggcgcccgtg cctctccctg cggtgcagtc
1921 cttgctagga gacgcacggt tttgccaaag agggacgctg tccacagccc tgcgactgga
1981 agtttttag gccctgcggt ggtagtggtg ttggtacggt tgtgtgcata agatgaacaa
2041 cgtttctctc aagacgagac tactagtatg ctgacggtgt gtgtatgtgg tggatggatt
2101 gtgccccgac catgaagagt gctgtgttgc ctcggcgctt ctgtcgccct ggatgtgcgt
2161 ggttccgaac gctggagtca tctgttgagg agcgagggtg ttgtcgggtc cgcccggcac
2221 ggccgcgtga tgtccggatg gggattgcga gcgagggcaa ccgcagcgca gatagcgccg
2281 cagcggatcg agctagcgca ggatgatgag agccgggcct tcgcggcgtg ggatcaggga
2341 ggagccaagg cggagtgcat gcgaggaaaa cagtgtgcgg caaagaacgg gctgcaagaa
2401 cgccttgcgc aaa

FIG. 2B

```
                     A             A        B
        .-250    .        .    .       .   .-200
        CGTCCTATTTTAATACTCCGAAGGAGGCAGTTGGCAGGCAACTGCCACTGACGTC
      31               31                  C
      .    B   .     .     .-150   .     .     .
CCGTAAGGGTAAGGGGACGTCCACTGGCGTCCCGTAAGGGGAAGGGGACGTAGGTACATAAATGTGCTAGGTAACTAAC

             C                            D
    .  .-100     .    .    .    .    .-50
GTTTGATTTTTTGTGGTATAATATATGTACCATGCTTTTAATAGAAGCTTGAATTTATAAATTAAAATATTTTTACAAT
                         S1
 D
        .      .            .       .     .
ATTTACGGAGAAATTAAAACTTTAAAAAAATTAACAT ATG ACA GCA ATT TTA GAA CGT CGT GAA AAT
  S1                                  Met Thr Ala Ile Leu Glu Arg Arg Glu Asn  10
                                                                          Ser

            .      .50          .           .           .           .
TCT AGC CTA TGG GCT CGT TTT TGT GAG TGG ATC ACT TCA ACT GAA AAC CGT TTA TAC ATC
Ser Ser Leu Trp Ala Arg Phe Cys Glu Trp Ile Thr Ser Thr Glu Asn Arg Leu Tyr Ile  30
Glu             Gly             Asn
         .100       .           .           .           .           .150
GGT TGG TTC GGT GTA ATC ATG ATC CCA TGT CTT CTT ACT GCA ACA TCA GTA TTC ATC ATC
Gly Trp Phe Gly Val Ile Met Ile Pro Cys Leu Leu Thr Ala Thr Ser Val Phe Ile Ile  50
                    Leu             Thr
         .          .           .           .          .-200        .
GCT TTC ATC GCT GCT CCG CCA GTA GAC ATC GAT GGT ATC CGT GAA CCA GTT TCA GGT TCT
Ala Phe Ile Ala Ala Pro Pro Val Asp Ile Asp Gly Ile Arg Glu Pro Val Ser Gly Ser  70
```

FIG. 3A

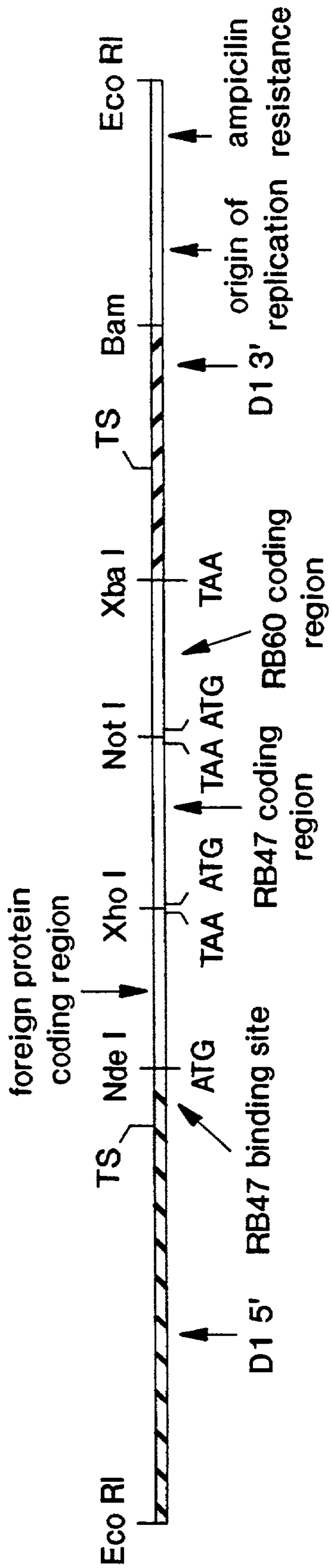
09/341550

```
            .           .           .               .250        .           .
CTT CTT TAC GGT AAC AAC ATC ATT ACA GGT GCT GTA ATC CCA ACT TCT AAC GCA ATC GGT
Leu Leu Tyr Gly Asn Asn Ile Ile Thr Gly Ala Val Ile Pro Thr Ser Asn Ala Ile Gly  90
                                Ser         Ile                 Ala

            .           .               .300        .           .           .
CTT CAC TTC TAC CCA ATT TGG GAA GCT GCT TCT CTA GAC GAG TGG TTA TAC AAC GGT GGT
Leu His Phe Tyr Pro Ile Trp Glu Ala Ala Ser Leu Asp Glu Trp Leu Try Asn Gly Gly 110
                                            Val

            .           .350        .               .           .           .
CCT TAC CAA CTT ATC GTT TGT CAC TTC CTT CTA GGT GTA TAC TGC TAC ATG GGT][CGT GAG
Pro Tyr Gln Leu Ile Val Cys His Phe Leu Leu Gly Val Tyr Cys Tyr Met Gly][Arg Glu 130
        Glu             Leu                         Ala

            .400        .           .               .           .           .450
TGG GAA TTA TCT TTC CGT TTA GGT ATG CGT CCA TGG ATC GCT GTA GCT TAC TCA GCT CCA
Trp Glu Leu Ser Phe Arg Leu Gly Met Arg Pro Trp Ile Ala Val Ala Tyr Ser Ala Pro 150

            .           .           .               .           .500        .
GTA GCT GCA GCT TCA GCT GTA TTC TTA GTT TAC CCT ATC GGC CAA GGT TCA TTC TCT GAC
Val Ala Ala Ala Ser Ala Val Phe Leu Val Tyr Pro Ile Gly Gln Gly Ser Phe Ser Asp 170
                Thr                 Ile

            .           .           .               .550        .           .
GGT ATG CCT TTA GGT][ATC TCT GGT ACT TTC AAC TTC ATG ATC GTA TTC CAA GCA GAA CAC
Gly Met Pro Leu Gly][Ile Ser Gly Thr Phe Asn Phe Met Ile Val Phe Gln Ala Glu His 190

            .           .           .600            .           .           .
AAC ATC CTT ATG CAC CCA TTC CAC ATG TTA GGT GTT GCT GGT GTA TTC GGT GGT TCA TTA
Asn Ile Leu Met His Pro Phe His Met Leu Gly Val Ala Gly Val Phe Gly Gly Ser Leu 210

            .           .850        .               .           .           .
TTC TCA GCT ATG CAC GGT TCT TTA GTT ACT TCA TCT TTA ATC CGT GAA ACA ACT GAA AAC
Phe Ser Ala Met His Gly Ser Leu Val Thr Ser Ser Leu Ile Arg Glu Thr Thr Glu Asn 230
```

FIG. 3B

```
                .700                                                    .750
GAA TCA GCT AAC GAA GGT TAC CGT TTC GGT CAA GAA GAA GAA ACT TAC AAC ATT GTA GCT
Glu Ser Ala Asn Glu Gly Tyr Arg Phe Gly Gln Glu Glu Glu Thr Tyr Asn Ile Val Ala 250

                                                                    .800
GCT CAT][GGT TAC TTT GGT CGT CTA ATC TTC CAA TAC GCT TCT TTC AAC AAC TCT CGT TCA
Ala His][Gly Tyr Phe Gly Arg Leu Ile Phe Gln Tyr Ala Ser Phe Asn Asn Ser Arg Ser 270

                                                        .850
TTA CAC TTC TTC TTA GCT GCT TGG CCG GTA ATC GGT ATT TGG TTC ACT GCT TTA GGT TTA
Leu His Phe Phe Leu Ala Ala Trp Pro Val Ile Gly Ile Trp Phe Thr Ala Leu Gly Leu 290
                                        Val                                 Ile

                                    .900
TCA ACT ATG GCA TTC AAC TTA AAC GGT TTC AAC TTC AAC CAA TCA GTA GTA GAC TCA CAA
Ser Thr Met Ala Phe Asn Leu Asn Gly Phe Asn Phe Asn Gln Ser Val Val Asp Ser Gln 310

                            .950
GGT CGT GTA CTA AAC ACT TGG GCA GAC ATC ATC AAC CGT GCT AAC TTA GGT ATG GAA GTA
Gly Arg Val Leu Asn Thr Trp Ala Asp Ile Ile Asn Arg Ala Asn Leu Gly Met Glu Val 330
            Ile
            .1000                                                          .1050
ATG CAC GAG CGT AAC GCT CAC AAC TTC CCT CTA GAC TTA GCT TCA ACT AAC TCT AGC TCA
Met His Glu Arg Asn Ala His Asn Phe Pro Leu Asp Leu Ala Ser Thr Asn Ser Ser Ser 350
                                                        Ala Ile Glu Ala Pro
                                                    .1100
AAC AAC TAA TTT TTTTTAAACTAAAATAAATCTGGTTAACCATACCTAGTTTATTTAGTTTATACACACTTTT
Asn Asn *Oc                                                S1
Thr     Gly     *Oc
                     .1150
CATATATATATACTTAATAGCTACCATAGGCAGTTGGCAGGACGTCCC
```

FIG. 3C



TS = transcription start and transcription stop

FIG. 4

```
  1 ATG GGC CAT CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT CGT  60
  1 M   G   H   H   H   H   H   H   H   H   H   H   S   S   G   H   I   E   G   R    20

 61 CAT ATG GCG ACT ACT GAG TCC TCG GCC CCG GCG GCC ACC ACC CAG CCG GCC AGC ACC CCG  120
 21 H   M   A   T   T   E   S   S   A   P   A   A   T   T   Q   P   A   S   T   P    40

121 CTG GCG AAC TCG TCG CTG TAC GTC GGT GAC CTG GAG AAG GAT GTC ACC GAG GCC CAG CTG  180
 41 L   A   N   S   S   L   Y   V   G   D   L   E   K   D   V   T   E   A   Q   L    60

181 TTC GAG CTC TTC TCC TCG GTT GGC CCT GTG GCC TCC ATT CGC GTG TGC CGC GAT GCC GTC  240
 61 F   E   L   F   S   S   V   G   P   V   A   S   I   R   V   C   R   D   A   V    80

241 ACG CGC CGC TCG CTG GGC TAC GCC TAC GTC AAC TAC AAC AGC GCT CTG GAC CCC CAG GCT  300
 81 T   R   R   S   L   G   Y   A   Y   V   N   Y   N   S   A   L   D   P   Q   A    100

301 GCT GAC CGC GCC ATG GAG ACC CTG AAC TAC CAT GTC GTG AAC GGC AAG CCT ATG CGC ATC  360
101 A   D   R   A   M   E   T   L   N   Y   H   V   V   N   G   K   P   M   R   I    120

361 ATG TGG TCG CAC CGC GAC CCT TCG GCC CGC AAG TCG GGC GTC GGC AAC ATC TTC ATC AAG  420
121 M   W   S   H   R   D   P   S   A   R   K   S   G   V   G   N   I   F   I   K    140

421 AAC CTG GAC AAG ACC ATC GAC GCC AAG GCC CTG CAC GAC ACC TTC TCG GCC TTC GGC AAG  480
141 N   L   D   K   T   I   D   A   K   A   L   H   D   T   F   S   A   F   G   K    160

481 ATT CTG TCC TGC AAG GTT GCC ACT GAC GCC AAC GGC GTG TCG AAG GGC TAC GGC TTC GTG  540
161 I   L   S   C   K   V   A   T   D   A   N   G   V   S   K   G   Y   G   F   V    180

541 CAC TTC GAG GAC CAG GCC GCT GCC GAT CGC GCC ATT CAG ACC GTC AAC CAG AAG AAG ATT  600
181 H   F   E   D   Q   A   A   A   D   R   A   I   Q   T   V   N   Q   K   K   I    200

601 GAG GGC AAG ATC GTG TAC GTG GCC CCC TTC CAG AAG CGC GCT GAC CGC CCC AGG GCA AGG  660
201 E   G   K   I   V   Y   V   A   P   F   Q   K   R   A   D   R   P   R   A   R    220
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FIG. 5A

09/341550


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661  ACG TTG TAC ACC AAC GTG TTC GTC AAG AAC TTG CCG GCC GAC ATC GGC GAC GAC GAG CTG  720
221  T   L   Y   T   N   V   F   V   K   N   L   P   A   D   I   G   D   D   E   L    240

721  GGC AAG ATG GCC ACC GAG CAC GGC GAG ATC ACC AGC GCG GTG GTC ATG AAG GAC GAC AAG  780
241  G   K   M   A   T   E   H   G   E   I   T   S   A   V   V   M   K   D   D   K    260

781  GGC GGC AGC AAG GGC TTC GGC TTC ATC AAC TTC AAG GAC GCC GAG TCG GCG GCC AAG TGC  840
261  G   G   S   K   G   F   G   F   I   N   F   K   D   A   E   S   A   A   K   C    280

841  GTG GAG TAC CTG AAC GAG CGC GAG ATG AGC GGC AAG ACC CTG TAC GCC GGC CGC GCC CAG  900
281  V   E   Y   L   N   E   R   E   M   S   G   K   T   L   Y   A   G   R   A   Q    300

901  AAG AAG ACC GAG CGC GAG GCG ATG CTG CGC CAG AAG GCC GAG GAG AGC AAG CAG GAG CGT  960
301  K   K   T   E   R   E   A   M   L   R   Q   K   A   E   E   S   K   Q   E   R    320

961  TAC CTG AAG TAC CAG AGC ATG AAC CTG TAC GTC AAG AAC CTG TCC GAC GAG GAG GTC GAC  1020
321  Y   L   K   Y   Q   S   M   N   L   Y   V   K   N   L   S   D   E   E   V   D    340

1021 GAC GAC GCC CTG CGT GAG CTG TTC GCC AAC TCT GGC ACC ATC ACC TCG TGC AAG GTC ATG  1080
341  D   D   A   L   R   E   L   F   A   N   S   G   T   I   T   S   C   K   V   M    360

1081 AAG GAC GGC AGC GGC AAG TCC AAG GGC TTC GGC TTC GTG TGC TTC ACC AGC CAC GAC GAG  1140
361  K   D   G   S   G   K   S   K   G   F   G   F   V   C   F   T   S   H   D   E    380

1141 GCC ACC CGG CCG CCC GTG ACC GAG ATG AAC GGC AAG ATG GTC AAG GGC AAG CCC CTG TAC  1200
381  A   T   R   P   P   V   T   E   M   N   G   K   M   V   K   G   K   P   L   Y    400

1201 GTG GCC CTG GCG CAG CGC AAG GAC GTG CGC CGT GCC ACC CAG CTG GAG GCC AAC ATG CAG  1260
401  V   A   L   A   Q   R   K   D   V   R   R   A   T   Q   L   E   A   N   M   Q    420

1261 GCG CGC ATG TAA GGATCC                                                           1278
421  A   R   M   *                                                                    424
```
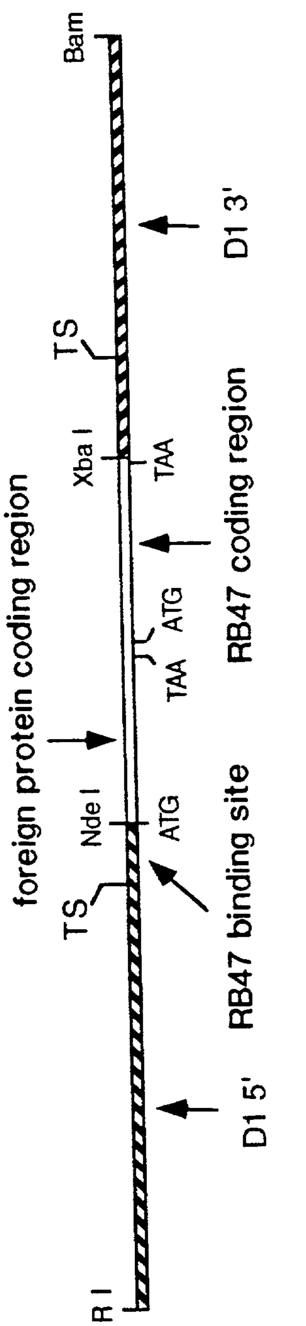

FIG. 5B



TS = transcription start and transcription stop

FIG. 6

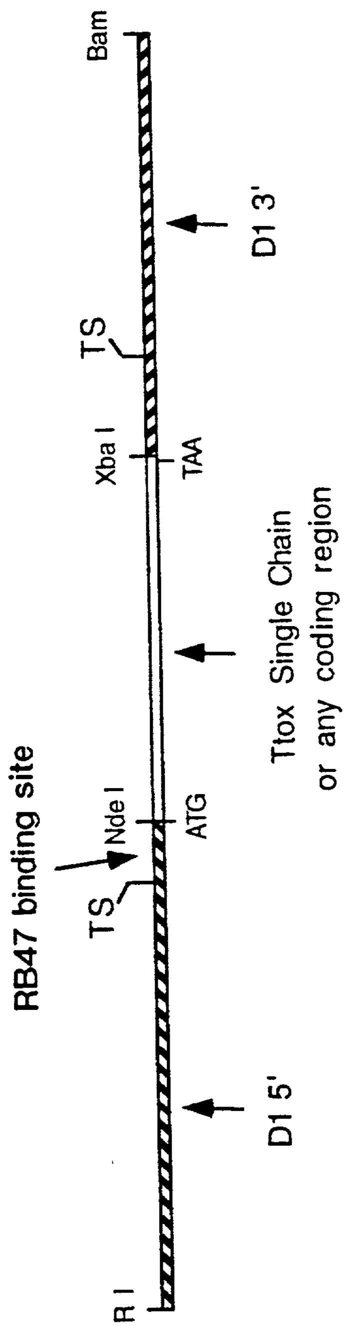


FIG. 7

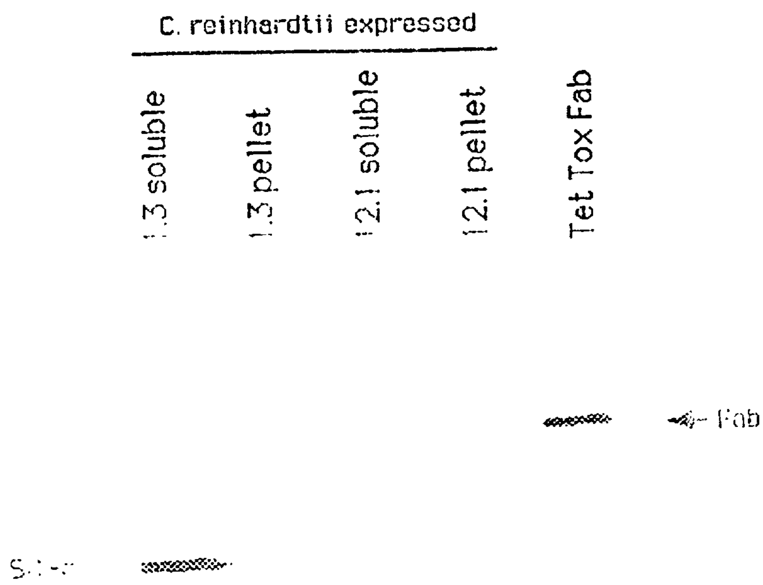
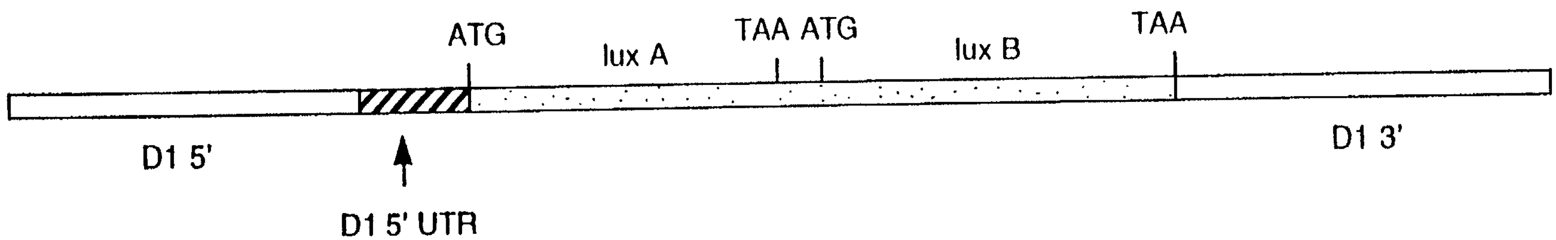

C. reinhardtii expressed
1.3 soluble
1.3 pellet
12.1 soluble
12.1 pellet
Tet Tox Fab
Fab

FIG. 8



Bacterial luciferase A and B proteins expressed from a single mRNA containing the psbA 5' UTR with translational activator element.

FIG. 9

09/341550

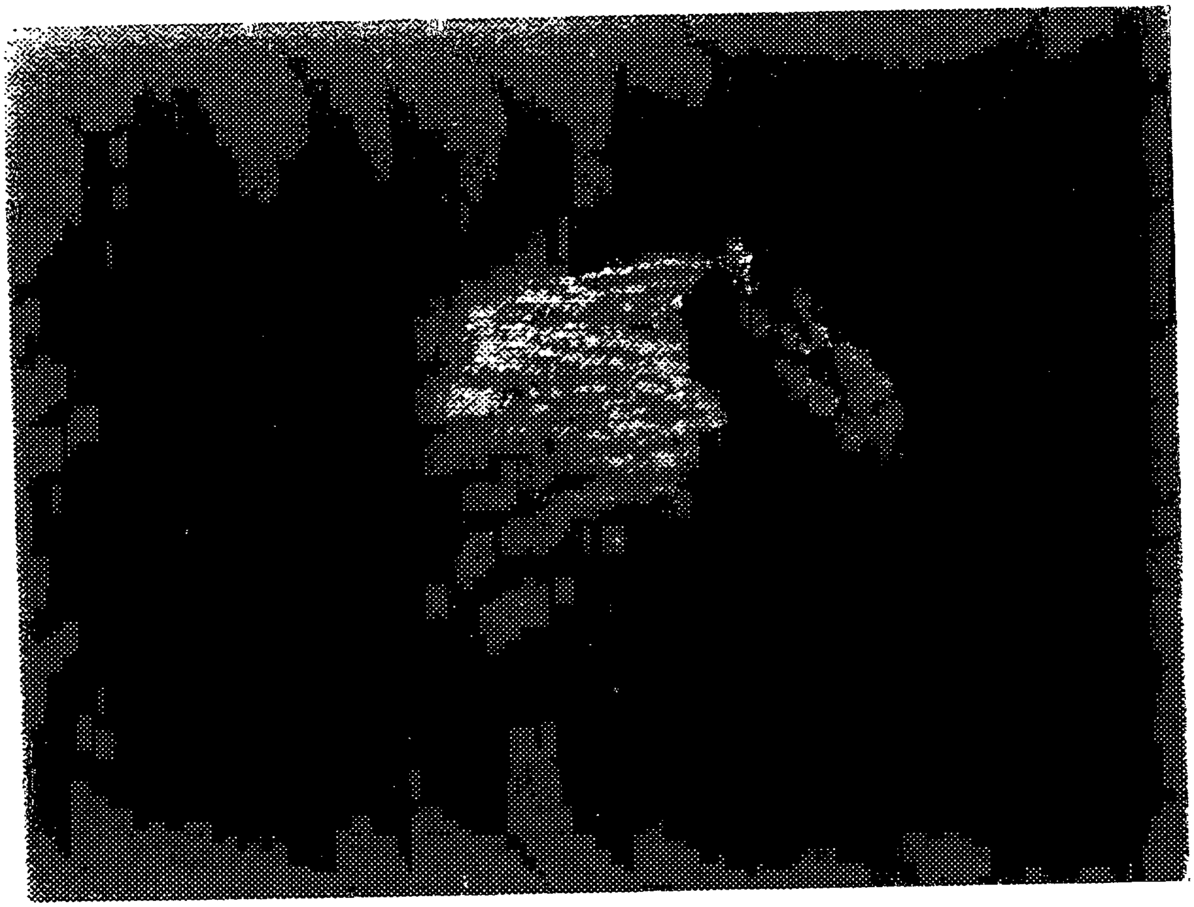


FIG. 10

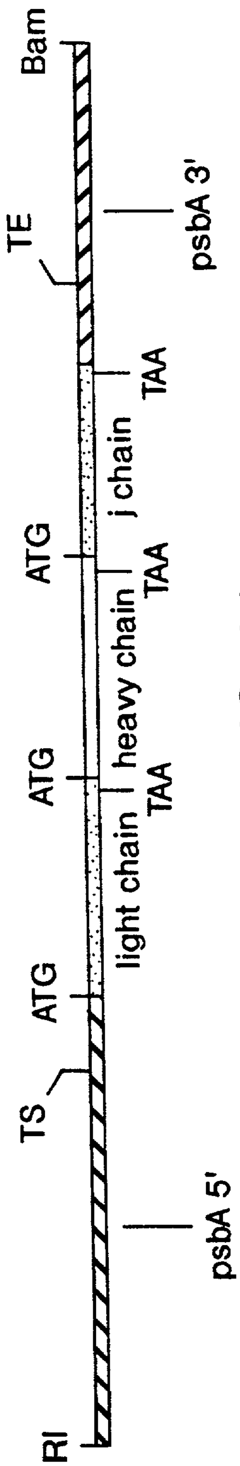

Bam
TE
psbA 3'
TAA
j chain
ATG
TAA
heavy chain
ATG
TAA
light chain
ATG
TS
psbA 5'
RI

FIG. 11

PATENT APPLICATION DECLARATION AND POWER OF ATTORNEY

I HEREBY DECLARE THAT:

My residence, post office address, and citizenship are as stated next to my name in PART A of page 2 hereof.

I believe I am the original, first, and sole inventor (if only one name is listed) or an original, first, and joint inventor (if plural names are listed) of the subject matter which is claimed and for which a patent is sought on the invention entitled RNA BINDING PROTEIN AND BINDING SITE USEFUL FOR EXPRESSION OF RECOMBINANT MOLECULES the specification of which:

___ is attached hereto
X was filed on January 16, 1998, as Application Serial No. PCT/US98/00840
and was amended on ____________ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Sec. 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Sec. 119 of any foreign application(s) for patent or inventor's certificate listed in PART B on page 2 hereof and have also identified in PART B on page 2 hereof any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

I hereby claim the benefit under Title 35, United States Code, Sec. 120 of any United States application(s) listed in PART C on page 2 hereof and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Sec. 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Sec. 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following as my attorneys or agents with full power of substitution to prosecute this application and transact all business in the United States Patent and Trademark Office connected therewith:

Douglas A. Bingham	Reg. No. 32,457	Thomas E. Northrup	Reg. No. 33,268
Thomas Fitting	Reg. No. 34,163	Emily Holmes	Reg. No. 40,652
Donald G. Lewis	Reg. No. 28,636		

whose mailing address for this application is:

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La Jolla, California 92037

See Page 2 attached, signed, and made a part hereof.

PATENT APPLICATION DECLARATION AND POWER OF ATTORNEY

PART A: Inventor Information And Signature

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Full name of SECOND joint inventor, if any ______
Citizenship ______ Post Office Address ______

Residence (if different) ______

Second Inventor's Signature: ______ Date: ______

Full name of THIRD joint inventor, if any ______
Citizenship ______ Post Office Address ______

Residence (if different) ______

Third Inventor's Signature: ______ Date: ______

Full name of FOURTH joint inventor, if any ______
Citizenship ______ Post Office Address ______

Residence (if different) ______

Fourth Inventor's Signature: ______ Date: ______

Full name of FIFTH joint inventor, if any ______
Citizenship ______ Post Office Address ______

Residence (if different) ______

Fifth Inventor's Signature: ______ Date: ______

PART B: Prior Foreign Application(s)

Serial No.	Country	Day/Month/Year Filed	Priority Claimed
			___ Yes ___ No

PART C: Claim For Benefit of Filing Date of Earlier U.S. Application(s)

Serial No.	Filing Date	Status:		
60/035,955	01/17/97	___ Patented	___ Pending	X Abandoned
60/069,400	12/12/97	___ Patented	___ Pending	X Abandoned

See Page 1 to which this is attached and from which this Page 2 continues.

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